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ORIGINAL PRE-CLINICAL SCIENCE

Effects of graft preservation conditions on coronary endothelium and cardiac functional recovery in a rat model of donation after circulatory death

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KEYWORDS:
heart transplantation;
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BACKGROUND: Use of cardiac grafts obtained with donation after circulatory death (DCD) could significantly improve donor heart availability. As DCD hearts undergo potentially deleterious warm ischemia and reperfusion, clinical protocols require optimization to ensure graft quality. Thus, we investigated effects of alternative preservation conditions on endothelial and/or vascular and contractile function in comparison with the current clinical standard.

METHODS: Using a rat DCD model, we compared currently used graft preservation conditions, St. Thomas n°2 (St. T) at 4°C, with potentially more suitable conditions for DCD hearts, adenosine-lidocaine preservation solution (A-L) at 4°C or 22°C. Following general anesthesia and diaphragm transection, hearts underwent either 0 or 18 min of in-situ warm ischemia, were explanted, flushed and stored for 15 min with either St. T at 4°C or A-L at 4°C or 22°C, and then reperfused under normothermic, aerobic conditions. Endothelial integrity and contractile function were determined.

RESULTS: Compared to 4°C preservation, 22°C A-L significantly increased endothelial nitric oxide synthase (eNOS) dimerization and reduced oxidative tissue damage ($p < 0.05$ for all). Furthermore, A-L at 22°C better preserved the endothelial glycocalyx and coronary flow compared with St. T, tended to reduce tissue calcium overload, and stimulated pro-survival signaling. No significant differences were observed in cardiac function among ischemic groups.

CONCLUSIONS: Twenty-two-degree Celsius A-L solution better preserves the coronary endothelium compared to 4°C St. T, which likely results from greater eNOS dimerization, reduced oxidative stress, and activation of the reperfusion injury salvage kinase (RISK) pathway. Improving heart preservation

Abbreviations: Akt, protein kinase B; A-L, adenosine-lidocaine cardioplegia; BH_4, tetrahydrobiopterin; BK, bradykinin; CD-138, syndecan-1; COX-2, cyclooxygenase 2; cTnI, cardiac troponin I; CVR, coronary vascular resistance; Cyt c, cytochrome c; DBD, donation after brain death; DCD, donation after circulatory death; dP/dtmax, maximum first derivative of left ventricular pressure; dP/dtmin, minimum first derivative of left ventricular pressure; eNOS, endothelial nitric oxide synthase; EPO, erythropoietin; FWIT, functional warm ischemic time; GTN, glyceryl trinitrate; H-FABP, heart-type fatty acid binding protein; L-NAME, N-omega-nitro-L-arginine methylester; NWI, no warm ischemia; NO, nitric oxide; RISK, reperfusion injury salvage kinase; ROS, reactive oxygen species; SAFE, survivor activating factor enhancement; SNP, sodium nitroprusside; St. T, St. Thomas n°2 cardioplegia

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conditions immediately following warm ischemia constitutes a promising approach for the optimization of clinical protocols in DCD heart transplantation.
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Heart transplantation with donation after circulatory death (DCD) may provide a solution to limitations in heart transplantation that result from lack of donor organs. Although heart transplantation remains the gold standard treatment for patients with end-stage heart failure,[1] the number of patients on heart transplantation waiting lists has increased over the last 2 decades, while the number of potential donors has remained stable, leading to a major organ shortage.[2,3] As an additional method to conventional donation after brain death (DBD), DCD could substantially improve the number of cardiac grafts available for transplantation[2] and potentially reduce mortality rates for patients on the heart waiting list by 40%.[4] Indeed, increases in cardiac transplant activity of 48% have been reported in the UK[5] and up to 15% in Australia[6] as a result of the implementation of DCD heart transplantation programs. Although DCD hearts undergo an inevitable period of warm ischemia followed by reperfusion (I/R), which raises concern for graft quality, more than 120 DCD hearts have been transplanted since 2014,[5,6] providing comparable short- and mid- term outcomes to DBD.[5,6]

In the context of DCD, it is critical to apply cardioprotective therapies at the onset of reperfusion as interventions to treat potential grafts in the donor are limited for ethical reasons. The majority of human DCD hearts are procured directly and flushed immediately after warm ischemia with the hyperkalemic preservation solution, St. Thomas n°2 cardioplegia (St. T), supplemented with erythropoietin (EPO) and glyceryl-trinitrate (GTN) for 3 to 6 min.[6-8] Erythropoietin is known to activate the reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement (SAFE) pathways, while glyceryl-trinitrate acts as a donor of nitric oxide, in addition to stimulating pro-survival kinases.[9] Hearts are then stored in hypothermic, non−perfused conditions from 15 to 30 min while preparations are made for perfusion in the Organ Care System (OCS™ Heart, Transmedics, Massachusetts, US).[6,8-12] Concerns about the use of hyperkalemic cardioplegic solutions and profound hypothermia have been raised, given that these conditions may exacerbate I/R injury in the context of DCD.[9,11,13,14] More specifically, it has been demonstrated that profound hypothermia and hyperkalemic cardioplegia are detrimental for the vascular endothelium.[13,14] Potential alternatives to the current preservation solutions, such as normokalemic adenosine-lidocaine cardioplegia (A-L), have been proposed.[11,13] Given that endothelial dysfunction is a key mediator of transplantation injury as it may result in the no-reflow phenomenon[15,16] or potentially lead to cardiac allograft vasculopathy,[17] there is an urgent need to optimize the initial reperfusion conditions to preserve the endothelium.[18] As few studies have investigated the specific effects of preservation solutions, such as adenosine-lidocaine and under conditions that do not fully represent the clinical DCD setting, further research is required to better characterize the effects and mechanisms of varying preservation conditions in the context of DCD.[11,13] Therefore, with the current study, we investigated the effects of graft preservation conditions, using A-L at tepid or cold temperatures, on endothelial and/or vascular and contractile function and the corresponding mechanisms in comparison with the current clinical standard (cold St. T) to help optimize clinical DCD heart transplantation protocols.

Methods

Ethics statement

All experiments were performed according to the European Convention for Animal Care and were approved by the Swiss animal welfare authorities and state veterinary office (Ethics Committee for Animal Experimentation [ECAE], Berne, Switzerland).

Experimental protocol

Simulation of withdrawal of life sustaining therapy and ischemia in situ

55 adult male Wistar rats (Janvier Labs, Le Genest-Saint-Isle, France), housed under standard conditions with unlimited access to food and water, were anesthetized intraperitoneally with 78 mg/kg ketamine (Narketan; Vetoquinol AG, Berne, Switzerland), 7.2 mg/kg xylazine (Xylapan; Vetoquinol AG, Berne, Switzerland) and 1.2 mg/kg acepromazine (Prequillan, Fatro AG, Bologna, Italy). Simulation of withdrawal of life sustaining therapy and in-situ ischemia were performed as described.[19,20] Briefly, withdrawal of life sustaining therapy (WLST) was simulated by asphyxiation following transection of the diaphragm. Functional warm ischemic time (FWIT) was considered to start when the peak systolic pressure dropped below 50 mm Hg and lasted either for 0 min (hearts were immediately explanted) or 18 min. Body temperature was maintained between 36.6 and 37.2°C during ischemia.

Preservation and storage conditions

After explantation, hearts were cannulated via the aorta on an ex-situ perfusion system. Four experimental groups were compared (Figure 1): A no-warm ischemia control (NWI) group (A) and an 18 min ischemia group (B) were flushed with cold (4°C) St. T preservation solution supplemented with 100 mg/L glyceryl trinitrate (GTN: Nitroglycerin Bioren; Sintetica, Mendrisio, Switzerland) and 5000 units/L erythropoietin (EPO: Eprex; Janssen, Berchem, Belgium).[21] In the third and fourth experimental groups, hearts underwent 18 min of ischemia and were then flushed with adenosine-lidocaine (A-L) solution at either 4°C (C) or at 22°C (D). All hearts were flushed for 3 min at a constant pressure of

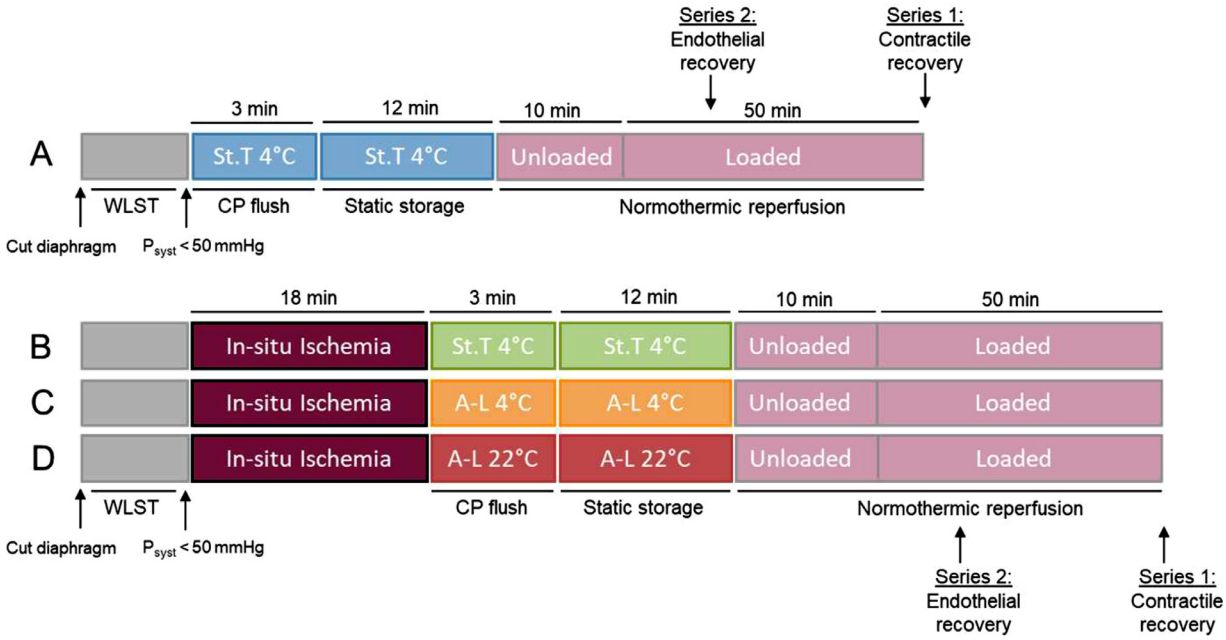

Figure 1 Experimental protocol: In a first perfusion series, hearts were subjected to either 0 (A) or 18 min (B-D) of in situ ischemia then hearts were explanted and subjected to differing cardioplegic flush and static storage conditions (Groups A and B: 4˚C St. Thomas n˚2 solution supplemented with glyceryl trinitrate and erythropoietin; Group C: 4˚C normokalemic adenosine-lidocaine cardioplegia or Group D: 22˚C normokalemic adenosine-lidocaine preservation solution). Afterward, all hearts were reperfused for 60 min and contractile recovery, circulating markers of cell death, signaling molecules, edema, eNOS coupling, inflammation markers, glycocalyx components, and oxidative stress were assessed. In a second perfusion series, endothelial function was assessed after 30 min reperfusion. A-L, normokalemic adenosine-lidocaine cardioplegia; CP, cardioplegic flush; P_{syst}, peak systolic pressure; St. T, St. Thomas n˚2 cardioplegia; WLST, simulated withdrawal of life sustaining therapy.

60 mm Hg, and then underwent 12 min static storage with immersion using the corresponding preservation conditions (flush solution and temperature).

Normothermic reperfusion

Two series of heart perfusions were generated after the static storage: one series was reperfused for 60 min to monitor post-ischemic contractile recovery and perform biochemical assays (cardiac recovery series, $n = 6$-8 rats per group), and the second series was reperfused for 30 min to assess endothelial function (endothelial recovery series, $n = 5$-8 rats per group). Both series were reperfused with modified Krebs-Henseleit buffer containing 1.25 mM Ca^{2+} and 11 mM glucose. For the first 10 min of reperfusion, hearts were perfused in the unloaded mode with a constant pressure of 60 mm Hg, and then switched to working mode with the minimum perfusion pressure set to 60 mm Hg. Throughout the reperfusion period, heart temperature was maintained at 37˚C and buffers gassed with 95% O_2 and 5% CO_2. At the end of the reperfusion period, ventricular tissue was quickly frozen in liquid nitrogen, and stored at -80˚C.

Data analysis

All results are reported as mean ± standard deviation. Statistical analyses were performed with GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Outlier values were identified with the Tukey test when data were outside the inner fences of the box and whiskers plot and removed from the analysis.[22] The Kruskal-Wallis test was performed for an overview of differences between experimental groups and, when significant, pairwise comparisons between groups were performed with Mann-Whitney tests. Correlations were analyzed using Spearman's rank correlation test. Two-tailed *p*-values were adjusted for multiple comparisons (modified, sequential, rejective Bonferroni procedure).[23] Corrected *p*-values are reported and considered statistically significant if <0.05.

Supplemental methods

The following methods are described in detail in the Supplementary material section online: endothelial and cardiac function evaluation, western blot, eNOS dimer determination, protein carbonylation, syndecan-1 tissue content, calcium content, tissue water content, cell death markers and mitochondrial damage, cytokine measurements, cardiac oxygen consumption and efficiency, mRNA expression, preservation solution components (Table S1) and primer sequences used in analyses of mRNA expression (Table S2).

Results

Endothelial-related parameters

Coronary vascular resistance (CVR) during the initial flush, coronary flow during reperfusion, and tissue glycocalyx content are shown in Figure 2. CVR during the initial flush was significantly increased in ischemic groups with St. T and A-L at 4˚C compared with the NWI group and with the

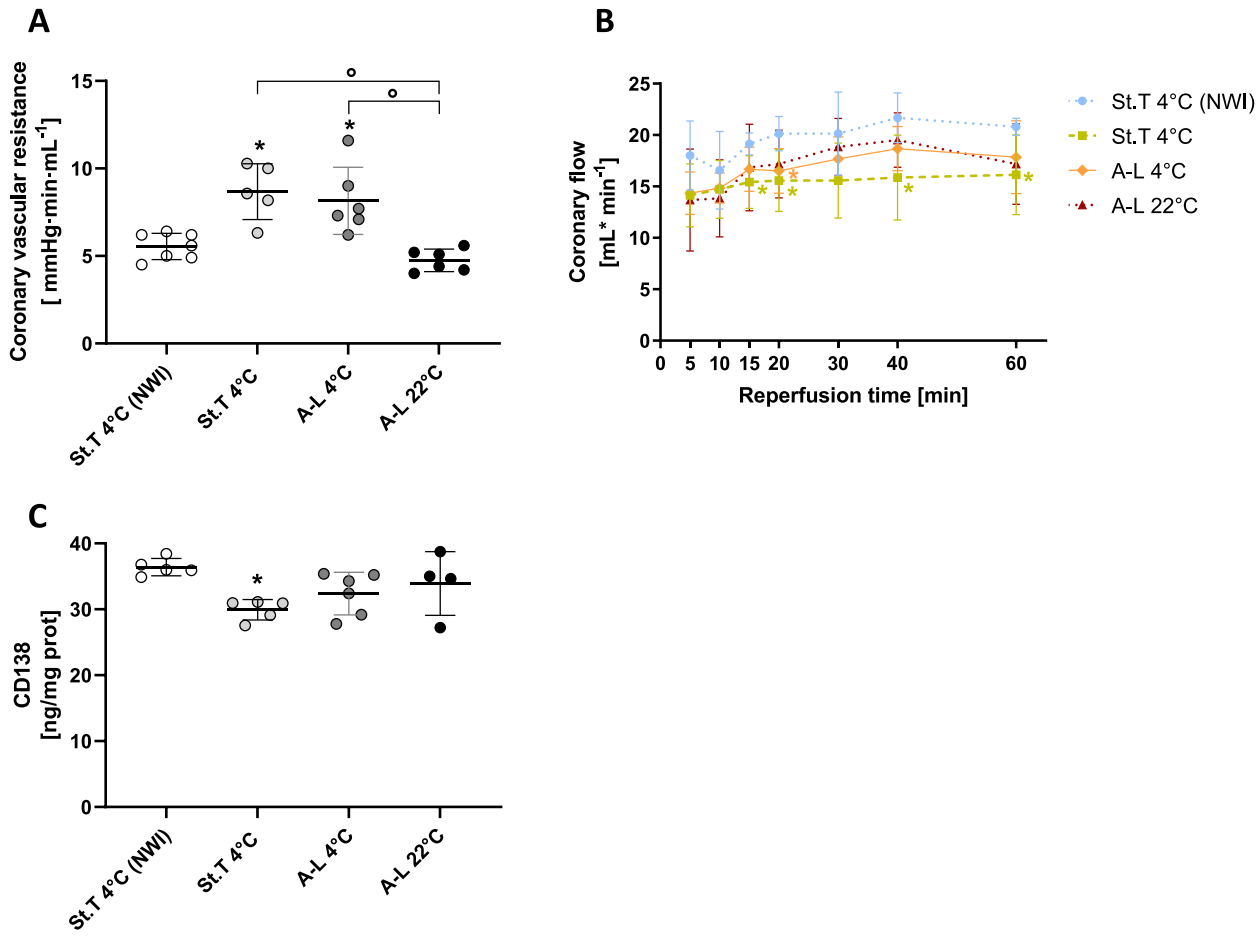

Figure 2 Endothelial-related parameters. (A) Coronary vascular resistance during the cardioplegic flush; (B) Post-ischemic coronary flow; (C) Tissue levels of glycocalyx component, syndecan 1(CD 138). *$p < 0.05$ vs St. T 4˚C (NWI), ˚$p < 0.05$. Data are expressed as mean ± SD; $n = 4$ to 7 per group. A-L, normokalemic adenosine-lidocaine solution; NWI, no warm ischemia; St. T, St. Thomas n˚2 solution.

ischemic group A-L at 22˚C ($p < 0.05$ for all). Coronary flow was significantly decreased in the ischemic group with St. T at 4˚C compared to the NWI group during loaded reperfusion ($p < 0.05$). Syndecan-1 (CD-138), a component of the glycocalyx, was measured in tissue after 60 min reperfusion. Syndecan-1 levels in ischemic hearts preserved with St. T at 4˚C were significantly lower compared to the NWI group, while hearts preserved with A-L maintained similar levels as the NWI group ($p < 0.05$).

eNOS dimerization and markers of oxidative damage

To evaluate endothelial nitric oxide synthase (eNOS) coupling, we measured the relative quantity of eNOS dimers after 60 min reperfusion, as shown in Figure 3A and B. We observed significantly more eNOS dimers in hearts with A-L at 22˚C compared with the other 2 ischemic groups ($p < 0.05$ for both). Total tissue oxidative stress, represented by protein carbonylation measured after 60 min reperfusion in Figure 3C, was significantly lower in the group with A-L at 22˚C compared with the other 2 ischemic groups preserved at 4˚C, ($p < 0.05$ for both), with levels similar to the NWI group. In addition, a representative protein carbonylation blot is shown in S6.

Calcium content

Calcium content, represented in Figure 4, was significantly increased in all ischemic hearts at 60 min reperfusion compared with the NWI group. Although among ischemic groups, no significant differences were observed; there was a tendency for higher calcium content in St. T at 4˚C compared to tepid A-L. Furthermore, tissue calcium content correlated negatively with post-ischemic cardiac function (left ventricular work) and with endothelial integrity (glycocalyx content), and positively with a marker of mitochondrial damage (Cyt c release; $p < 0.05$ for all).

Activation of key signaling molecules

Western blot analyses of phosphorylated (p) and total protein kinase B (Akt) and eNOS, analyzed after 60 min of reperfusion, are presented in Figure 5. No significant

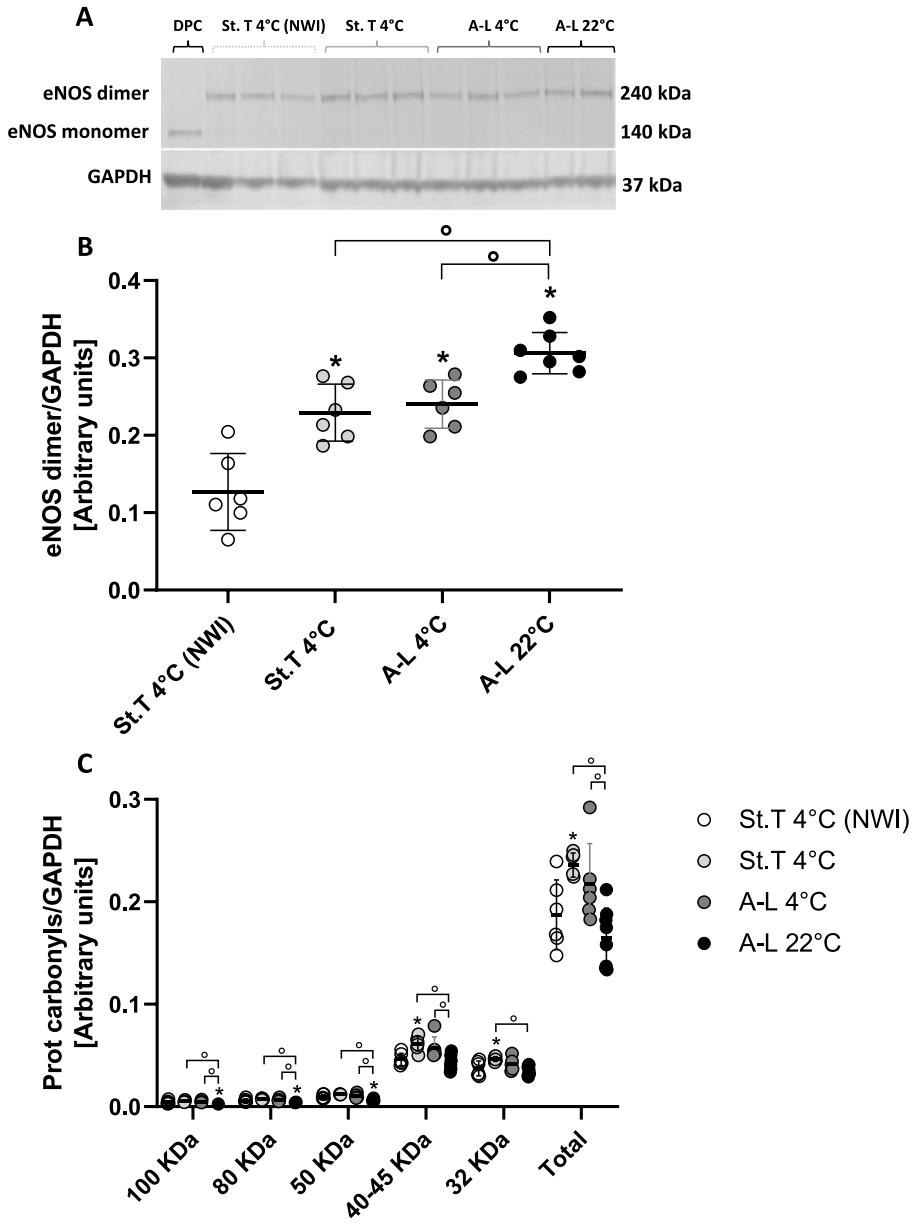

Figure 3 eNOS dimerization and oxidative stress. (A) Representative eNOS dimer western blots; (B) eNOS dimer content; (C) Protein carbonylation indicating oxidative protein damage. *$p < 0.05$ vs St. T 4°C (NWI), °$p < 0.05$. Data are expressed as mean ± SD; $n = 6$ to 7 per group. A-L, normokalemic adenosine-lidocaine solution; DPC, denatured protein control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; kDa, kilodalton; NWI, no warm ischemia; Prot, protein; St. T, St. Thomas n°2 solution.

differences were observed for p-eNOS expression. p-Akt was significantly higher in all ischemic hearts compared to the NWI group and in ischemic hearts with 22°C A-L compared with St. T ($p < 0.05$ for all).

Post-ischemic functional recovery

Absolute values of cardiac function are presented in Figure 6. Left ventricular work and cardiac output were significantly lower for all ischemic groups compared to the NWI group (St. T at 4°C; $p < 0.05$ for all). No differences were observed among ischemic groups. At 60 min reperfusion, dP/dtmax was significantly decreased only in the group with St. T at 4°C compared with the non−ischemic group ($p < 0.05$). In general, dP/dtmin recovered less well in all ischemic hearts compared with NWI hearts.

Markers of cell death and mitochondrial damage

Release of cardiac troponin I (cTnI), heart-type fatty acid binding protein (H-FABP) and cytochrome c (Cyt c) are presented in Figure 7. For all markers, concentrations at 60 min reperfusion were significantly higher in all ischemic groups compared with the NWI group ($p < 0.05$ for all). Although levels of cell death markers tended to be lower in the A-L at 22°C group compared to other ischemic groups, no statistically significant differences were observed among ischemic groups.

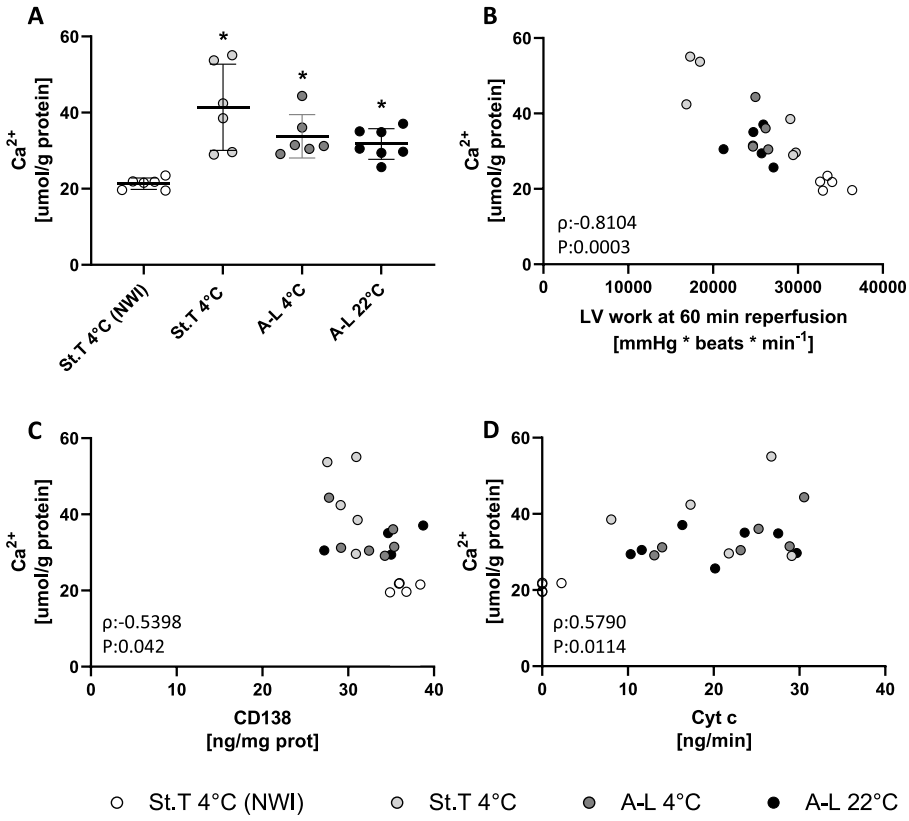

Figure 4 Calcium content. (A) Calcium content in heart tissue; (B) Correlation between calcium content and left ventricular (LV) work at 60 min reperfusion; (C) Correlation between calcium content and tissue CD138 (syndecan-1); (D) Correlation between calcium content and Cyt c release. *$p < 0.05$ vs St. T 4˚C (NWI). Data are expressed as mean ± SD and correlations data are expressed as Spearman's rho (ρ); $n = 6$ to 7 hearts per group. A-L, normokalemic adenosine-lidocaine solution; Cyt c, cytochrome c; NWI, no warm ischemia; St. T, St. Thomas n˚2 solution.

Supplemental results

The following results are described in detail in the Supporting information section available online: coronary vascular function (Figure S1), tissue water content (Figure S2), mRNA expression of genes involved in inflammation after 60 min reperfusion (Figure S3), tissue protein expression of inflammatory markers (Figure S4), oxygen consumption and efficiency (Figure S5), representative protein carbonylation blot (Figure S6) and measurements during in-situ and/or withdrawal phase in the series 1 of hearts (Table S3).

Discussion

Protective interventions at the onset of reperfusion are of particular importance for minimization of I/R injury in cardiac grafts obtained with DCD. In this study, we demonstrate that tepid (22˚C) A-L preservation better preserves cardiac grafts, particularly endothelial function, compared to cold A-L or St. T, as demonstrated by greater eNOS coupling (Figure 8). A significant reduction in oxidative stress was observed in hearts preserved with tepid A-L, which could contribute to the increase in eNOS dimers. Furthermore, tepid, A-L preservation better preserves the endothelial glycocalyx, as indicated by the retention of syndecan-1, whereas a significant loss was observed in hearts preserved with cold St. T vs NWI hearts. As expected, tissue calcium content tended to be higher in hearts preserved with St. T compared with A-L at 22˚C, as St. T is hyperkalemic. Although both St. T and A-L solutions included supplements to activate the RISK pathway, stimulation appeared more effective with tepid A-L than cold St. T, as demonstrated by increased phosphorylation of Akt. Taken together, reduced calcium content, and oxidative stress, in combination with the increased recruitment of the cardioprotective RISK pathway, likely contribute to improved graft preservation provided with tepid, A-L preservation.

Coronary flow during loaded reperfusion was significantly lower in ischemic hearts preserved with St. T at 4˚C compared to the NWI group, while that of hearts with A-L was not different compared to the NWI group. Although hypothermia after a period of warm global ischemia has been shown to increase endothelial injury,[13,14,24] it is unlikely to be entirely responsible for reduced coronary flow, as coronary flow in the 4˚C A-L group was not significantly different than NWI and almost identical to that in hearts with tepid A-L. Rather, high potassium content in hearts preserved with St. T after exposure to a period of warm ischemia in combination with glycocalyx shedding (as described below) may result in impaired endothelial function.[14]

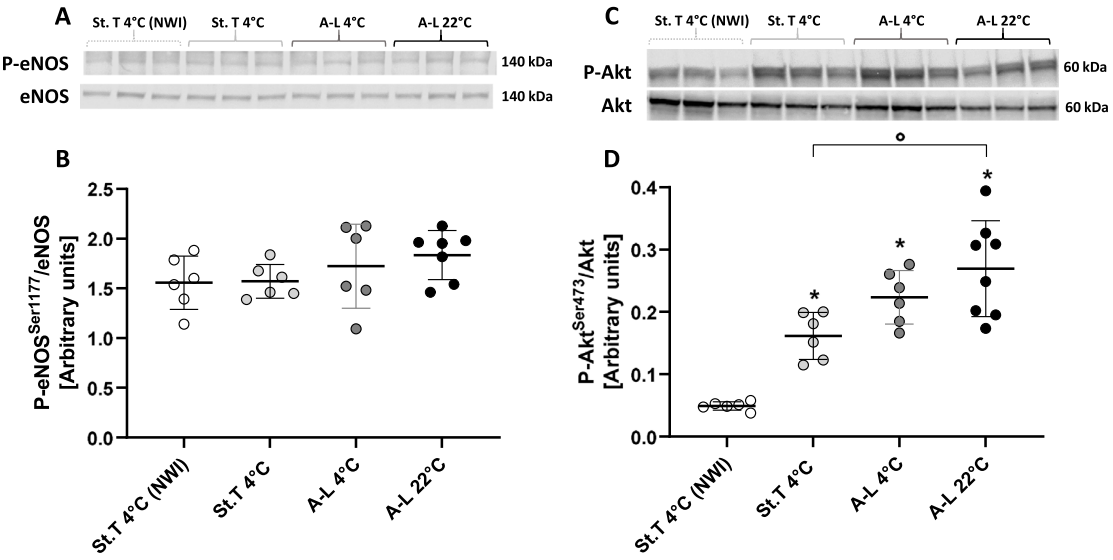

Figure 5 Activation of key signaling molecules. (A) Representative western blots for phosphorylated (Ser 1177) and total eNOS proteins; (B) Ratios of phosphorylated (Ser 1177) to total proteins for eNOS, (C) Representative western blots for phosphorylated (Ser 473) and total Akt proteins (D) Ratios of phosphorylated (Ser 473) to total proteins for Akt, $*p < 0.05$ vs St. T. 4°C (NWI), $^{\circ}p < 0.05$, Kruskal Wallis analysis reported no significant differences for Panel A (p-value: 0.22). Data are expressed as mean ± SD; $n = 6$ to 8 per group. A-L, normokalemic adenosine-lidocaine solution; kDa, kilodalton; NWI, no warm ischemia; St. T, St. Thomas n°2 solution.

Tepid, normokalemic A-L preservation promotes eNOS dimerization compared to cold A-L or St. T in our DCD model in parallel with less oxidative stress, indicating better eNOS coupling, and endothelial integrity. Consequently, reduced coronary flow during reperfusion in ischemic hearts with St. T likely stems from lower nitric oxide (NO) production as a result of reduced dimer preservation. Under physiologic conditions, NO is mainly produced by eNOS, which converts L-arginine into NO and L-citrulline in the presence of oxygen, calmodulin, tetrahydrobiopterin (BH_4),

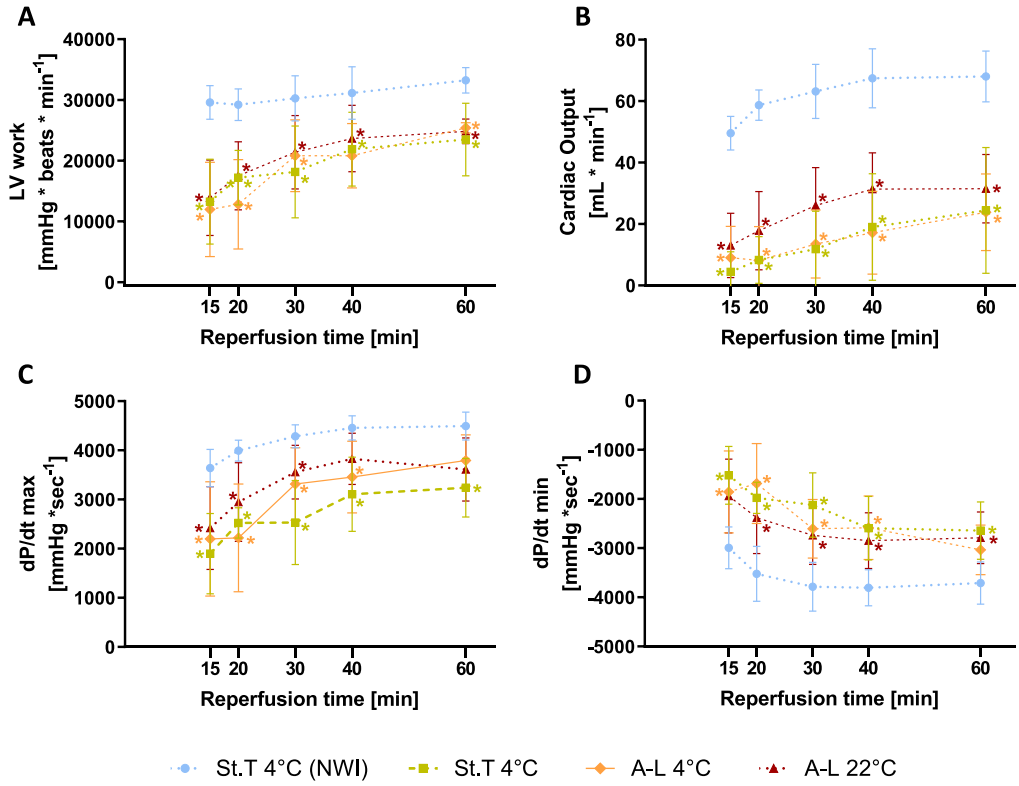

Figure 6 Post-ischemic cardiac function. (A) LV work (left ventricular work: heart rate*developed pressure); (B) CO (cardiac output); (C) dP/dtmax (maximum first derivative of LV pressure); (D) dP/dtmin (minimum first derivative of LV pressure). $*p < 0.05$ vs St. T 4°C (NWI). Data are expressed as mean ± SD; $n = 6$ to 8 per group. A-L, normokalemic adenosine-lidocaine solution; NWI, no warm ischemia; St. T, St. Thomas n°2 solution.

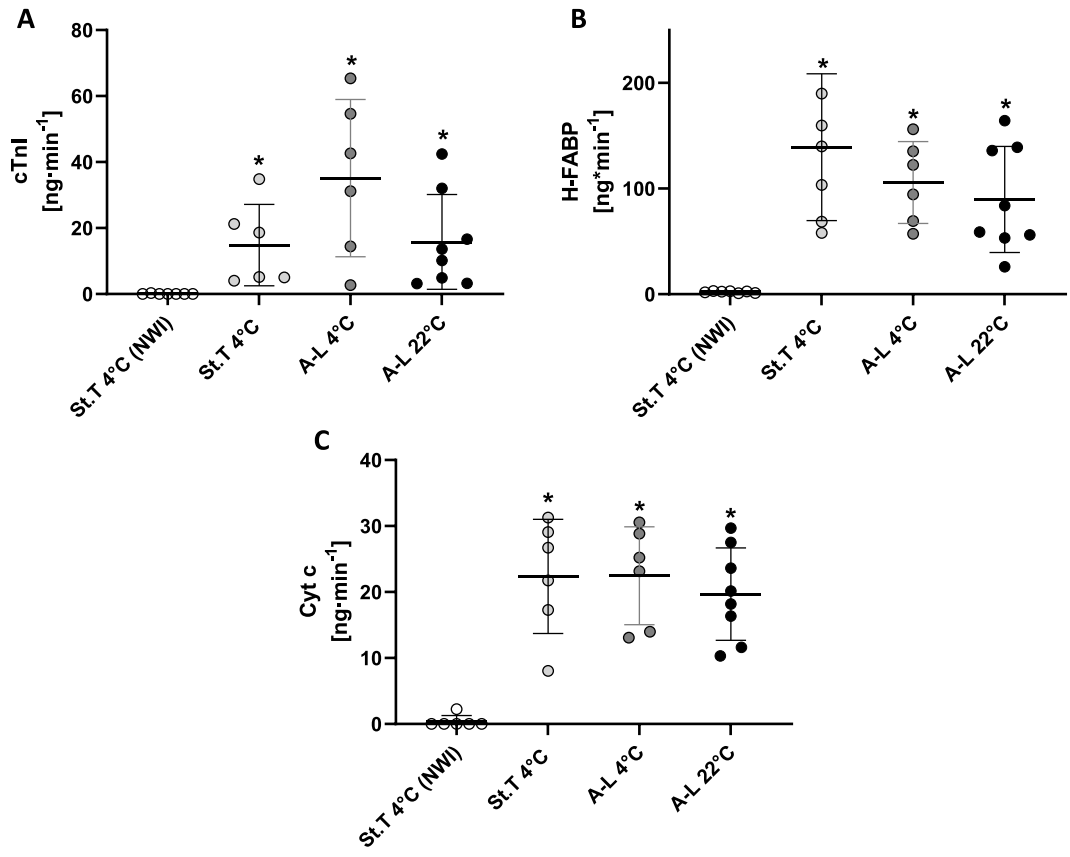

Figure 7 Release of markers of cell death and mitochondrial damage at 60 min reperfusion. (A) Release of cardiac troponin I (cTnI); (B) Release of heart-type fatty acid binding protein (H-FABP) and (C) Release of cytochrome c (Cyt c). *$p < 0.05$ vs St. T 4°C (NWI). Data are expressed as mean ± SD; $n = 6$ to 8 per group. A-L, normokalemic adenosine-lidocaine solution; NWI, no warm ischemia; St. T, St. Thomas n°2 solution.

flavin mononucleotide, flavin adenine dinucleotide, and NADPH.[25] One of the main causes of impaired endothelial function after I/R is the lack of availability of NO.[26,27] I/R induces depletion of BH_4 levels as a result of its oxidation and/or reduced synthesis, which provokes eNOS uncoupling.[28] When eNOS is uncoupled, the disturbed electron flux leads to the production of superoxide (O^{2-}) instead of NO.[28] In turn, O^{2-} can react with NO producing peroxynitrite, thereby creating a vicious cycle of exacerbated eNOS uncoupling through further BH_4 oxidation and dimer destabilization.[29] eNOS dimerization is required for NO production. Thus, given their greater dimer content, hearts with tepid A-L should have greater NO production and reduced superoxide and peroxynitrite production compared with the other ischemic hearts. This is in agreement with our findings for lower oxidative stress in hearts preserved with A-L at 22°C compared with other ischemic hearts. Interestingly, for ischemic hearts preserved with A-L, eNOS coupling was significantly greater at 22°C compared to 4°C. The reason for this difference is not immediately apparent. Although all hearts with A-L received reduced glutathione and mannitol[30] to limit reactive oxygen species (ROS) production and promote eNOS coupling, it appears that this approach is more effective at warmer temperatures as demonstrated by our protein carbonylation results. Interestingly, although we could observe greater eNOS dimer preservation in hearts subjected to A-L at 22°C, no differences in eNOS phosphorylation were observed among groups. This pattern was observed as well by Bibli et al. when they examined the effects of GTN on eNOS.[31]

In addition, ischemic hearts preserved with St. T at 4°C suffered from glycocalyx shedding which could contribute to the reduction in coronary flow. Our marker for cardiac glycocalyx levels, syndecan-1, remained similar in NWI hearts and hearts with A-L, but was significantly reduced with St. T at 4°C. The glycocalyx plays an important role in preserving endothelial cell integrity and is required for proper eNOS function.[32] Furthermore, it is one of the first structures to sustain damage as consequence of I/R.[33,34] Indeed, I/R promotes glycocalyx disruption, leading to edema, increased coronary perfusion pressure, and impaired endothelial-dependent vasodilation.[35,36]

Ischemic hearts preserved with St. T at 4°C tended to have a higher tissue calcium content compared with hearts preserved with A-L. In fact, calcium content correlated positively with cardiac dysfunction and mitochondrial damage, which could be expected, as hyperkalemic cardioplegia can potentially exacerbate Ca^{2+} overload and increase reperfusion injury.[14] In addition, calcium content correlated positively with glycocalyx degradation, which is in agreement with Jackson-Weaver et al.,[37] who observed that I/R injury

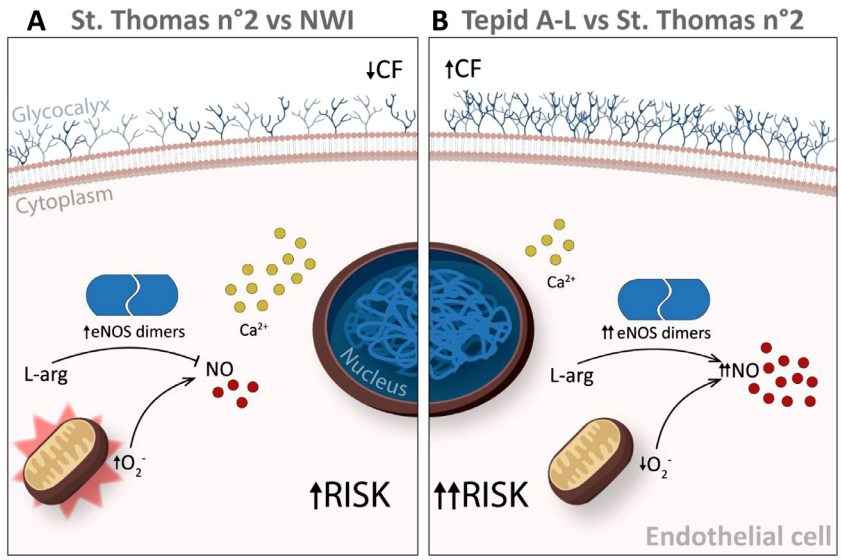

Figure 8 Proposed effects of preservation conditions on coronary endothelium. (A) Current clinical preservation conditions (St. Thomas n°2 at 4°C) following warm ischemia result in higher tissue calcium content, which promotes mitochondrial damage, leading to higher oxidative stress compared with hearts not exposed to warm ischemia preserved under the same conditions (NWI). Although an increase in eNOS dimers and RISK pathway activation could promote greater nitric oxide production in ischemic hearts compared to NWI, increased levels of reactive oxygen species (as observed) will decrease NO availability by both reacting with available nitric oxide (NO) to produce peroxynitrite and promoting NOS uncoupling. Reduced NO, together with decreased glycocalyx content, likely contributes to reduced coronary flow upon normothermic reperfusion. (B) Compared to current clinical preservation conditions (St. Thomas n°2 at 4°C), preservation with tepid adenosine-lidocaine (A-L at 22°C) tends to reduce tissue calcium content, which likely contributes to the significantly reduced oxidative stress. This lower oxidative stress, in combination with significant increases eNOS dimerization, promote greater NO production. Increased NO, in parallel with better preservation of the endothelial glycocalyx likely contributes to improved coronary flow during normothermic reperfusion. Furthermore, preservation with A-L solution increased pro-survival (RISK) signaling compared with St. T, which may also contribute to superior cardiac graft preservation.

promoted Ca^{2+} release from endothelial cells, which led to glycocalyx damage in cultured cells.

Tepid A-L preservation leads to a greater activation of prosurvival pathways compared with St. T. We measured a significantly higher Akt phosphorylation in ischemic A-L 22°C hearts compared with St T at 4°C hearts, indicating a stronger activation of the RISK pathway. Interestingly, although both preservation solutions have components that activate prosurvival kinases[9,38-41]; St. T was less effective than A-L in our study. Another potential cardioprotective pathway could be mediated by the cyclooxygenase 2 (COX-2). COX-2 can induce inflammation and apoptosis[42,43] in the human heart. However, recent studies demonstrated that expression of COX-2 confers cardioprotection in mouse hearts exposed to I/R.[44,45] In our study, we observed that COX-2 mRNA expression was induced in hearts subjected to ischemia (Fig. S2, D) with a tendency for higher levels in hearts subjected to A-L 22°C. COX-2 mRNA levels in our study followed the same pattern as p-Akt. Although it is not yet known if there is connection between these 2 molecules in the heart, Akt regulates COX-2 mRNA expression in cancer cells.[46]

The release of markers of cell death and mitochondrial damage were significantly increased in all ischemic groups compared to the non−ischemic group. We observed a tendency for lower cTnI in the hearts preserved with A-L at 22°C in comparison with A-L at 4°C. This finding was in agreement with those in a porcine model of DCD for which lower endothelial and myocyte injury as well as lower cTnI release was reported in hearts preserved with A-L solution at 25°C compared to 5°C.[13] In addition, cytochrome c tended to be higher in hearts preserved at 4°C, which is in agreement with other studies reporting a higher release of damage-associated molecular patterns in endothelial cells subjected to cold storage and warm reperfusion.[47]

We demonstrated a significantly greater NO-dependent pre-vasodilation in ischemic hearts compared to NWI hearts with the measured NOS-dependent CVR upon infusion of the NOS inhibitor (L-NAME). Thus, caution is needed in the interpretation of our vascular function results in response to BK and SNP. This pre-dilation likely results from vasodilators present in the preservation solutions. Although both vasodilators GTN and adenosine are NO-independent, they may also possess NO-dependent characteristics.[31,48] In contrast, although the NWI hearts were also treated with St. T containing GTN, pre-vasodilation response was significantly lower.

Although our findings indicate that the current clinical approach in DCD heart transplantation may be improved by optimization of the composition and conditions of application of the preservation solution, additional research is required to make evidence-based recommendations for future protocol development. In the current study, we report that use of tepid preservation solution was associated with reduced oxidative stress and improved endothelial recovery compared with cold preservation solution Recently, we demonstrated that use of hypothermic, oxygenated perfusion (HOPE) improved cardiac recovery compared to cold

static storage.[19] One of the protective effects from HOPE may be related to NO generation during HOPE due to the presence of oxygen and the reduction of oxidative stress. Given that protective interventions before ischemic onset are limited for ethical reasons in DCD, and that much I/R injury occurs in the first minutes of reperfusion, the precise conditions and composition of the preservation solution applied immediately after warm ischemia takes on great importance. Ultimately, a balance combining the optimal composition, temperature, and oxygen will likely be required for optimal graft preservation.

This study has several limitations. The DCD model we used does not take into account neurologic injury, which is frequently present in DCD donors, and could potentially lead to remote preconditioning. In our DCD model, WLST was simulated through an asphyxiation by dissecting the rat diaphragm, which could lead to a shorter period of time between WLST, and circulatory arrest compared with human DCD donors. In addition, in this rat model of DCD, heparinization was required to prevent clot formation, and consequently achieve an optimal reperfusion. However, in some countries the use of antemortem heparin is not allowed. Our hearts were reperfused using a crystalloid buffer and not a blood-based perfusate as in the clinical scenario and hearts were not transplanted. Lastly, although we believe that St. T no. 2 may be detrimental to DCD hearts, this has only been demonstrated with our findings at 4°C, further research would be required to confirm these results at other temperatures.

In this study, we demonstrate that the use of tepid A-L preservation improved eNOS coupling, glycocalyx preservation, and activation of pro-survival pathways while reducing oxidative stress compared with the hyperkalemic preservation solution currently use in DCD protocols. Applying cardioplegic solutions developed for DBD conditions is unlikely to be ideal for graft preservation in DCD, as hearts are subjected to warm ischemia, and arrested before procurement. Understanding the physiologic status of the heart at the time of procurement is critical for the development of optimal cardioprotective therapies in DCD. DCD grafts are submitted to a period of warm ischemia that leads to depletion of energy stores (ATP), anaerobic metabolism causing intracellular acidosis,[11,49] and reperfusion may cause further damage through the rapid restoration of the extracellular pH, calcium overload, ROS generation, mitochondrial permeability transition pore opening and activation of cell death pathways.[50] In current, clinical DCD protocols, hearts are generally reperfused and stored briefly with cold, hyperkalemic cardioplegia (St. T). It is known that hyperkalemic cardioplegia could exacerbate Ca^{2+} overload and lead to increased reperfusion injury.[14] Furthermore, in DCD conditions, cold cardioplegia has been reported to provoke greater endothelial and myocyte injury than tepid conditions.[13] As such, room for improvement exists with current clinical protocols in DCD heart transplantation such that reperfusion injury could be limited or even prevented. Careful selection of preservation conditions, taking into consideration the physiologic changes occurring during DCD and mechanisms of injury upon reperfusion, should help optimize graft quality, and permit the expansion of DCD heart transplantation in a safe manner.

Author contributions

NMC contributed to all aspects of this manuscript including study planning and design, model development, performing experiments, data collection and analysis, and preparation of the manuscript. RW, MA, AS, NK, and AJ participated in performing experiments, data collection and preparation of the manuscript. TC participated in data analysis and preparation of the manuscript. SL participated in study planning and design, model development, data analysis, and preparation of the manuscript. All authors contributed to the article and approved the submitted version.

Disclosure statement

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Supplementary materials

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Supplementary data

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